A possible new oxidation marker for hair adulteration: detection of PTeCA (1H-pyrrole-2,3,4,5-tetracarboxylic acid) in bleached hair

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Abstract

Hair analysis has become a valuable tool in forensic toxicology to assess drug or alcohol abstinence. Yet, hair adulteration by cosmetic products presents a major challenge for forensic hair analysis. Oxidative treatments, e.g. bleaching, may lead to analyte loss and thereby to false negative results. Currently, the eumelanin degradation product 1H-pyrrole-2,3,5-tricarboxylic acid (PTCA) serves as a marker for oxidative hair treatment, but requires the definition of cut-off values. To investigate further eumelanin degradation products as markers for oxidative hair treatment, hair samples with and without *in vitro* bleaching (hydrogen peroxide (H_2O_2) concentrations 1.9 % up to 12 %; incubation times 15 min, 30 min, 60 min) were analyzed by liquid chromatography coupled to high-resolution time of flight mass spectrometry (HPLC-HRMS). The distribution of eumelanin degradation products along the hair shaft was investigated for routine applicability after segmentation of cosmetically untreated hair samples and authentically treated hair samples. The signals of the eumelanin degradation products PTCA, 1H-pyrrole-2,3,4-tricarboxylic acid (isoPTCA) and 1H-pyrrole-2,3,4,5-tetracarboxylic acid (PTeCA) were found to be significantly elevated after *in vitro* bleaching already with low H_2O_2 concentrations and after short incubation times. In contrast to PTCA and isoPTCA, PTeCA was not detectable in cosmetically untreated segments up to 12 cm from hair root and was only formed through the oxidation process. The results of the study show that the detection of PTeCA within the proximal 3 to 6 cm segment can be applied to reliably detect hair adulteration attempts through hair bleaching.

Keywords: PTeCA, PTCA, oxidative hair treatment, hair biomarker, melanin degradation

1. Introduction

Hair analysis has become an important tool to retrospectively monitor consumption behavior of alcohol or drugs of abuse in forensic toxicological laboratories. Analysis of hair is therefore routinely applied for the assessment of abstinence control (e.g. drugs of abuse, ethanol) in the context of driving ability examination or workplace drug testing[1]. Associated with serious consequences, such as e.g. loss of driving license or loss of workplace, adulteration attempts to achieve negative test results are not unusual and present a major challenge in forensic hair analysis. To circumvent positive test results, easily accessible, typical adulteration treatments include "detox" shampoos, perming, dyeing, thermal hair straightening, permanent waves and bleaching[2-6]. Especially oxidative treatments with hydrogen peroxide (H_2O_2) in alkaline media (e.g. bleaching or permanent coloring) significantly affect measured analyte concentrations in hair and might lead to false negative results[7-9]. This practice results in a large variety of different treatment conditions complicating the interpretation of analysis results especially if cosmetic treatment is in doubt and respective information is not enclosed.

In routine hair analysis, one approach to address this problem is the visual inspection prior to analysis. Another approach is the observation of colored extracts of dyed hair samples after organic extraction. However, bleached hair samples usually lead to almost colorless extracts and a subjective visual control stays ineffective. Therefore, to objectively distinguish between untreated and bleached hair samples, alternative approaches for the differentiation have been studied. They include the detection of bleached hair samples with infrared (IR) spectroscopy[10], with fluorescence microscopy[11] or after proteolytic digestion[12]. Yet, those techniques are normally not routinely applied or available in forensic laboratories.

Only recently, Petzel-Witt *et al.* identified 1H-pyrrole-2,3,5-tricarboxylic acid (PTCA), an eumelanin degradation product, as a marker for oxidative hair treatment by using liquid-chromatography tandem-mass spectrometry (LC-MS/MS)[13]. Their studies revealed that PTCA concentrations significantly increased after *in vitro* bleaching of hair samples. However, with PTCA being measurable already in untreated hair samples, the definition of cut-off values for routine applications is indispensable. Hence, a robust marker for oxidative treated hair that is exclusively formed through the adulteration process would be desirable.

Melanin is the pigment which gives rise to the natural human hair color and is composed of two melanin forms. Eumelanin is responsible for the black to brown hair color, whereas pheomelanin gives a reddish-brown color shade[14,15]. Eumelanin is composed of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) which are further oxidized to the eumelanin polymer[16]. PTCA is the main eumelanin degradation product and is formed through photodegradation or alkaline oxidation of melanin[17]. From several studies about melanin degradation products, it is known that not only PTCA, but also 1H-pyrrole-2,3-dicarboxylic acid (PDCA), 1H-pyrrole-2,3,4-tricarboxylic acid (isoPTCA) and 1H-pyrrole-2,3,4,5-tetracarboxylic acid (PTeCA) are formed during alkaline H_2O_2 oxidation[14,16,18,19] through direct oxidation of DHI- or DHICA-melanin or cross-linking of DHI moieties[16].

The aim of the present study was to investigate more deeply the above mentioned eumelanin degradation products in oxidatively treated hair samples within the framework of an untargeted hair metabolomics project. First studies have been conducted to study their general usefulness and applicability to serve as potential markers for oxidative hair treatment.

2. Materials and methods

2.1 Chemicals and reagents

PTCA was obtained as analytical reference standard from ChemPur (Karlsruhe, Germany). Heavy-labeled and deuterated internal standards (IS) adenosine ribose-D_1, arginine-$^{13}C_6$, caffeine 3-methyl-^{13}C, carnitine trimethyl-D_9, deoxycholic acid-D_4, D-fructose ^{13}C, hippuric acid ^{15}N, leucine-D_{10}, lysine-D_4, phenylalanine-D_1, proline ^{15}N and tryptophane-D_5 were purchased from Cambridge Isotope Laboratories, Inc (Andover, MA, USA) which were delivered by ReseaChem Life Science (Burgdorf, Switzerland) or Sigma-Aldrich (Buchs, Switzerland). Water, acetonitrile (ACN), dichloromethane (DCM) and methanol (MeOH) of HPLC grade were purchased from Fluka (Buchs, Switzerland). All other chemicals used were from Merck (Zug, Switzerland) and of the highest grade available.

Commercial shampoo (I am, Intense Moisture, Buchs, Switzerland) was purchased from a local store and bleaching reagents (Venice Oxydant & active lotion containing 1.9 %, 3 %, 6 %, 9 % or 12% H_2O_2; Venice bleaching oxidation powder) were purchased from DOBI (Suhr, Switzerland).

2.2 Hair samples

Cosmetically untreated and treated hair samples were received after anonymization from the Center for Forensic Hair Analytics (Zurich, Switzerland) from routine case work and from volunteers from our lab. All samples were analyzed after anonymization in full conformance with Swiss laws (statement of Cantonal Ethics Board of the Canton of Zurich: BASEC-Nr. Req-2017-00946). Hair was sampled close to the scalp and stored in aluminum foil at room temperature until further analysis. Investigation of hair samples was categorized in four experiments: experiment 1 (investigation of oxidative treatment and hair color distribution),

experiment 2 (investigation of influence of H_2O_2 concentrations and incubation times), experiment 3 (untreated and treated, authentic hair samples) and experiment 4 (segmentation of untreated hair samples). In *experiment 1*, untreated hair samples (n=21) were assigned to four hair color classes (black (n = 4); dark brown to medium brown (n = 7); light brown to dark blond (n = 5); and medium blond to light blond (n = 5)). The proximal 3 cm hair segments of each of these hair strands were either left untreated or bleached with 9 % H_2O_2 (see section 2.3). In *experiment 2*, hair samples (n = 3, analysis of the proximal 5 cm segment) were divided into several strands and investigated under different H_2O_2 concentrations and incubation times (see section 2.3). Within *experiment 3*, untreated (n = 12, analysis of the proximal 3 cm segment) and visibly treated, authentic hair samples (n = 2 with self-reported bleaching within the last 3 months; n = 11 obviously treated hair samples with no record about cosmetic hair treatment) were included (see section 3.4). Thereof, 8 hair samples with untreated proximal segments and cosmetically treated distal segments were analyzed in a paired manner. Finally, *experiment 4* (see section 3.5) included cosmetically untreated hair samples (n = 3, sampled in mid-May 2019) with a total hair length of up to 18 cm, segmented into 3 cm segments.

2.3 General bleaching procedure

Cosmetically untreated hair strands (n = 21, experiment 1) were each divided into two portions. One portion of the hair strand was left untreated, the other portion was bleached *in vitro* using a mixture of 2 g bleaching oxidase (containing 9 % H_2O_2) and 1 g bleaching oxidation powder, according to the manufacturer's instructions. They were incubated for 30 min at room temperature and consequently rinsed under running water until the pH of the washing solution was neutral. To investigate the effect of H_2O_2 concentrations and incubation

times on the PTCA, isoPTCA and PTeCA content, hair strands (n = 3, experiment 2) were each bleached *in vitro* with a mixture of 2 g bleaching oxidase (containing 1.9 %, 3 %, 6 %, 9 % or 12 % H_2O_2) and 1 g bleaching oxidation powder and incubated during 15 min, 30 min or 60 min at room temperature, again in direct comparison of paired, untreated hair strands. After the hair adulteration, both untreated and bleached hair strands were rinsed under running water followed by rinsing with 500 µL shampoo solution (1 mL shampoo, 9 mL H_2O), again followed by rigorous rinsing under running water until the wash solution was clear. Hair samples were dried overnight at room temperature and prepared as described under section 2.4.

2.4 Sample preparation

Untreated and treated hair samples were prepared according to a sample preparation protocol described by Eisenbeiss *et al.*[20], with minor changes. Briefly, hair samples were consecutively washed with DCM, acetone, H_2O and acetone and dried overnight. An average of 30 mg hair was pulverized and extracted with 1 mL ACN/H_2O (2:8, *v/v*) and 20 µL IS solution (adenosine ribose-D_1 0.075 mM, arginine-$^{13}C_6$ 1.5 mM, caffeine 3-methyl-^{13}C 1 mM, carnitine trimethyl-D_9 0.5 mM, deoxycholic acid-D_4 0.009 mM, D-fructose ^{13}C 0.6 mM, hippuric acid ^{15}N 2.5 mM, leucine-D_{10} 1.5 mM, lysine-D_4 3.5 mM, phenylalanine-D_1 1.5 mM, proline ^{15}N 3.5 mM and tryptophan-D_5 1.25 mM) during incubation in an ultrasonic bath for 16 h. Subsequently, samples were centrifuged (5 min, 9'000 rpm) and the supernatant (900 µL) was transferred into a pill glass and evaporated to dryness under nitrogen (N_2) at 35 °C. The dried extract was reconstituted in 250 µL ACN/H_2O (2:8, *v/v*), followed by combined centrifugation and filtration (5 min, 9'000 rpm, VWR centrifugal filter, 0.45 µm pore size, VWR, Dietikon, Switzerland). Additionally, a solvent blank was

prepared in the same manner and pooled quality control (QC) samples were obtained by mixing together 30 µL of each filtrate.

2.5 HPLC-HRMS analysis

Analysis of all samples was performed on a Thermo Fischer Ultimate 3000 UHPLC system (Thermo Fischer Scientific, San Jose, CA) coupled to a high-resolution (HR) time-of-flight (TOF) instrument system (TripleTOF 6600, Sciex, Concord, Ontario, Canada) as described in detail by Steuer *et al.*[21]. Chromatographic separation was achieved on a Waters (Baden-Daettwil, Switzerland) XSelect HSST RP-C18 column (150 mm x 2.1 mm i.d.; 2.5 µm particle size) using 10 mM ammonium formate buffer in water containing 0.1 % (*v*/*v*) formic acid (eluent A) and MeOH containing 0.1 % (*v*/*v*) formic acid (eluent B) for gradient elution within a total run time of 20 min. The applied LC gradient was as follows: 1 min 100 % eluent A; 1 – 15 min increase to 100 % eluent B and held for 3 min; 18.01 min decrease to start conditions and re-equilibration for 2 min.

Within one analysis, HR mass spectra (MS) and tandem mass spectrometry (MS/MS) data was acquired using negative electrospray ionization (ESI) mode with two methods in two separate runs: TOF-MS only and information-dependent acquisition (IDA). MS analysis was performed with a DuoSpray ion source at a resolving power (full width at half maximum (fwhm) at *m/z* 400) of 30'000 in MS and 30'000 in MS2 (HR mode) or 15'000 (high-sensitivity mode). Automatic calibration was done using an atmospheric-pressure chemical ionization (APCI) negative calibration solution (Sciex) every 3 samples.

Conditions for the TOF-MS method were as follows: mass range *m/z* 50 to *m/z* 1000, accumulation time 100 msec, collision energy (CE) 5 eV. IDA scans (accumulation time 100 msec, CE 35 eV with a CE spread of 15 eV) were performed over a mass range of *m/z* 50 to

m/z 1000. Further criteria for IDA experiments were dynamic background subtraction on the four most intense ions, intensity threshold above 100 counts per second (cps) and exclusion time of 5 sec (half peak width) after two occurrences in high sensitivity mode. Acquisition of all MS parameters was controlled by Analyst TF software 1.7 (Sciex, Concord, Ontario, Canada).

All samples were analyzed in randomized order. A sequence of blanks and pooled QC samples was injected at the sequence beginning to allow instrument equilibration. Additionally, a pooled QC sample was injected every five samples to monitor the analyticalperformance over the whole batch. For this, retention time shifts and mean coefficient of variation (CV) of peak areas of all three eumelanin degradation products and internal standards were checked. For targeted data evaluation, analyte peak areas of precursor ions ($[M-H]^-$) were obtained by peak integration of data obtained from TOF-MS runs using MultiQuant V 2.1 (Sciex, Concord, Ontario, Canada). MS/MS spectra acquired by IDA acquisition were used for metabolite identification only. For semi-quantitative interpretation, peak areas were normalized by the respective sample weight. Direct comparison of hair sample results was only performed with samples from the same batch.

2.6 Statistical evaluation

Statistical comparison of untreated and treated hair samples was performed in GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) using a paired t-test. An ordinary one-way ANOVA was chosen to compare the distribution of PTCA, isoPTCA and PTeCA over the different hair colors. Authentic samples were compared using the Wilcoxon matched pairs signed rank test for paired analysis and the non-parametrical Mann-Whitney-U test.

3. Results

3.1 Identification of PTCA, isoPTCA and PTeCA and analytical procedure

Within the untargeted acquired data sets of experiments 1-4, targeted searches for PTCA, isoPTCA and PTeCA were performed by accurate precursor mass searching (precursor extraction window: 5ppm). MS/MS spectra obtained by information-dependent acquisition of PTCA and PTeCA and corresponding structural formulas and fragmentation patterns are shown in Figure 1. As fragmentation of isoPTCA is the same as for PTCA, the presentation of the MS/MS spectra for isoPTCA was omitted. The identity of PTCA was unambiguously confirmed by comparison of retention time and mass spectra of a reference compound. Unfortunately for PTeCA and isoPTCA, no reference standards were commercially available. However, based on the accurate precursor mass ($M\text{-}H^-$) and the interpretation of the fragmentation pattern, PTeCA and isoPTCA were finally identified resulting in an identification level 2 as suggested by the Metabolomics Standard Initiative (MSI)[22]. Respective chromatograms for the three analytes under different experimental conditions are presented in Figure S1 of the supplementary information.

Mean coefficient of variations (CV) were calculated for the three analytes from pooled QC samples measured throughout the batches. For all three analytes, mean CV's were < 15 % in every case. Therefore, data was considered to be of good quality and was hence used for data analysis.

3.2 Effect of oxidative treatment and hair colors on PTCA, isoPTCA and PTeCA content

Untreated, natural hair samples (proximal 3 cm segment) from 21 individuals were analyzed without any cosmetic treatment and after *in vitro* bleaching with 9 % H_2O_2 during 30 min

(*experiment 1*). Results are shown in Figure 2. PTCA and isoPTCA were detectable in all untreated hair samples. In contrast, PTeCA could not be detected in any of the untreated hair samples. After *in vitro* bleaching, PTCA and isoPTCA signals significantly increased ($p < 0.0001$) and PTeCA was detectable with high abundance. Between the four hair color classes, no significant difference could be observed for PTCA and isoPTCA in untreated hair samples (Figure S2 of the supporting information). Yet, after bleaching, a significantly lower amount of PTCA, isoPTCA and PTeCA was detected in blond and light-brown hair samples compared to black hair samples.

3.3 Influence of H_2O_2 concentrations and incubation times on PTCA, isoPTCA and PTeCA content

Results of the influence of H_2O_2 concentrations and incubation times at room temperature on PTCA, isoPTCA and PTeCA contents (each normalized to its respective sample weight) are given in Figure 3 (*experiment 2*). PTeCA/PTCA ratios in dependence of H_2O_2 concentrations and incubation times can be found in Figure S3 of the supporting information.

The increase of abundance of the three analytes after bleaching was statistically significant for all incubation times and bleaching conditions (determination of significance level with ordinary one-way ANOVA, $p < 0.05$: significant). PTeCA was already detectable after 15 min of incubation time and using the lowest H_2O_2 concentration (1.9 %). A time- and H_2O_2 concentration-dependency was observed for PTCA, isoPTCA and PTeCA. For all three analytes and an incubation time of 15 min, an increase of the analyte content was observed reaching a maximum with a concentration of 9 % H_2O_2. For incubation during one hour, tendencies towards an abundance increase with increasing H_2O_2 concentrations could be observed for PTeCA, while the PTCA content stayed almost constant regardless of the H_2O_2

concentration used. PTeCA/PTCA ratios mainly increased with increasing incubation time. An increase observed with rising H_2O_2 concentrations was primary observed in relation to incubation during one hour.

3.4 Authentic hair samples - applicability

To assess the applicability of the identified eumelanin degradation products as markers for oxidative hair treatment to authentic samples, untreated hair samples and hair samples that were visibly treated with cosmetic products were analyzed (*experiment 3*). PTeCA could be detected in 12 out of 13 treated segments, but remained undetectable in all untreated hair samples (see Figure 4). Within this sample group, 8 hair samples with untreated proximal hair segments and treated distal segments were analyzed for changes of PTCA, isoPTCA and PTeCA content (see Figure S4 in the supplementary information). Changes for PTCA and isoPTCA contents were not statistically significant, whereas PTeCA content again significantly increased in treated hair segments ($p < 0.05$).

3.5 Detectability of PTCA, isoPTCA and PTeCA along untreated hair strands

To analyze the natural prevalence and distribution of the eumelanin degradation products PTCA, isoPTCA and PTeCA along the hair shaft in cosmetically untreated hair samples, hair from 3 individuals (up to 18 cm hair length) who reported no cosmetic hair treatment were analyzed (*experiment 4*). Results after segmentation of the hair strands in 3 cm segments are shown in Figure 5. PTeCA remained undetectable in all subjects in the first four segments (0-12 cm from hair root) and was only detectable in trace amounts up from 12 cm distance from hair root and in only two subjects. In contrast, PTCA and isoPTCA were detectable in all hair segments from the first proximal 3 to 6 cm segment to the hair tip. Even in one (PTCA) and

two (isoPTCA) of the very first segments (0-3 cm), the corresponding eumelanin degradation products could be detected, respectively. Both analyte signals increased with increasing distance from the hair root.

4. Discussion

4.1 Targeted analysis and interpretation of PTCA, isoPTCA and PTeCA under oxidative conditions

To objectively distinguish between cosmetically untreated and bleached hair samples, robust biomarkers for routine implementation are required. Previous studies identified the eumelanin degradation product PTCA as a marker for oxidative hair treatment, however requiring the consideration of cut-off values[13]. Within the present, preliminary study, the analysis of further eumelanin degradation products, their formation under different conditions and the applicability as markers for oxidative hair treatment was studied. *In vitro* studies were performed with the proximal 3 cm segment, being the segment the most prone to manipulation attempts. LC-MS/MS, a standard analytical procedure in forensic laboratories[23], was applied as the implementation into routine methods for hair analysis is desirable. At best, detection of adulteration attempts should be possible within the very same run as routine analytes.

A targeted data evaluation for eumelanin degradation products was performed within an untargeted acquired data set. This means that the applied analytical method was not developed specifically for melanin degradation products. Instead, it is a universal method to detect as much small molecules as possible including eumelanin degradation products in a biological sample (in this case, in hair samples). The method is part of a bigger research project investigating metabolomics in hair. As such, absolute quantification was not aimed

for and is not necessary in case of an adulteration marker that is either completely missing or present in high concentrations after bleaching.

As usual for an untargeted metabolomics MS method, general considerations from a classical analytical method validation are not included (e.g. influence of matrix effects). A potential influence of such effects cannot be fully excluded. In the case where a marker is completely missing in one condition and only formed in the other, this should be negligible. However, further studies are required for the implementation into routine hair analysis.

While PTCA, isoPTCA and PTeCA were satisfactorily detected, the other known eumelanin degradation product PDCA could not be detected neither in untreated nor in bleached hair samples. It is known that alkaline H_2O_2 conditions lead to generally lower PDCA concentrations than PTCA concentrations in human hair[14,24] so that these low levels of PDCA might not be detectable with the applied analytical procedure.

After *in vitro* bleaching of hair samples with 9 % H_2O_2 during 30 min, a significant increase of PTCA and isoPTCA signals was observed which is in line with studies published by Petzel-Witt *et al.*[13] who found a significant increase of PTCA in hair samples treated with 9 % H_2O_2 solution in alkaline media and incubation for 40 min. However, both analytes were also detected in untreated hair samples, leading to the necessity of cut-off values if application to routine procedures is intended. To differentiate between untreated and oxidatively treated hair samples, a biomarker that is not present in untreated hair samples but is only formed through the adulteration process would be better. Thereby, no quantitative method is needed and the determination of cut-off values could be avoided; simple detection of this marker in a hair sample would be a strong indication for hair manipulation in case bleaching is (not yet) suspected. These characteristics were observed for PTeCA which was

not detectable in the proximal 3 cm segment of untreated samples, but only after the *in vitro* bleaching which strongly suggests a new formation through the oxidative treatment (see Figure 2).

Regarding the distribution of PTCA and isoPTCA over the four hair color classes (black, dark brown to medium brown, light brown to dark blond, and medium blond to light blond), varying low levels of both analytes were found in untreated hair samples with no significant difference between the hair color classes. Though, after the bleaching process, a clear dependency on the hair color, being directly correlated with the eumelanin content[14,15,25], was observable (see Figure S2 of the supplementary information). Also for PTeCA, this distribution over the hair colors was observed after *in vitro* bleaching already showing a clear formation of PTeCA even in light blond, bleached hair samples. This correlation according to the eumelanin content additionally supports its origin from eumelanin degradation. Even though PTeCA was only formed through the oxidation process, its eumelanin dependency could be a limitation for its applicability to e.g. pure white hair samples, which contain no melanin pigments[26]. However, in the vast majority of cases, the detection of PTeCA would indeed be a valuable decision support.

4.2 Influence of H_2O_2 concentrations and incubation times on PTCA, isoPTCA and PTeCA contents

Commercially available products for hair dyeing and bleaching contain H_2O_2 for oxidation and alkalinizing agents for better hair penetration[27]. Common H_2O_2 concentrations range from 2 % to 12 % and are applied during variable incubation times depending on the desired brightening results, typically for 20-40 min[27,28]. In practice, different dyeing or bleaching

conditions could affect the detectability and the power of the adulteration markers which is why a wide range of bleaching conditions was investigated within our study.

The obtained results showed that PTCA, isoPTCA and PTeCA were detectable with a significant signal increase already using the shortest incubation time and the lowest H_2O_2 concentration (see Figure 3). In theory, an increase of eumelanin degradation products towards a plateau would be expected as a consequence of complete eumelanin oxidation. As shown in our study, pushing the incubation time to 60 min or using concentrations of 12 % H_2O_2 does not further increase the formation of PTCA. In contrast, a downward trend under these maximum conditions is observable. These findings could firstly be due to a completed oxidation of melanin during 60 min already with low H_2O_2 concentrations. Secondly, the beginning decrease of PTCA, isoPTCA and PTeCA contents could be due to further, unspecific secondary reactions of H_2O_2 or other ingredients.

Apart from this, the bleaching process can be regarded as an "accelerated hair aging process" as observed with the increased PTeCA/PTCA ratio. This finding is comparable to studies from Ito *et al.*. Therein, the authors propose the increased PTeCA/PTCA ratio as a marker for eumelanin aging. They showed that synthetic eumelanin is unstable at higher temperatures (heating up to 100 °C over 18 or 40 °C over 180 days) with rapid decarboxylation and enhanced cross-linking (aging)[16]. This is why a possible new formation of PTeCA under excessive thermal hair straightening should be evaluated in future studies.

4.3 Applicability to authentic hair samples and detectability of PTCA, isoPTCA and PTeCA along untreated hair strands

To test the applicability of the results obtained under controlled *in vitro* conditions, untreated and treated authentic hair samples were analyzed for PTCA, isoPTCA and PTeCA.

Furthermore, to check for a possible new formation of PTeCA without oxidative hair treatment and thus exclude a misinterpretation of PTeCA findings, longer natural hair samples were segmented into 3 cm segments and the longitudinal distribution of PTCA, isoPTCA and PTeCA was investigated.

Except for one hair sample, PTeCA could be detected in all cosmetically treated segments. (see Figure 4 and Figure S4 of the supplementary information). Only for two treated hair samples, the proximal 3 cm segment, being directly comparable to the *in vitro* studies, were available and provided a record of bleaching within this time frame. The other authentic samples were chosen from visual classification as "cosmetically treated and presumably bleached". Treated segments were located in segments with differing distance from the hair root. As PTeCA was not detected in any untreated hair sample within the proximal 3 cm segment, detection of PTeCA in the bleached proximal segments can only be explained through formation due to the oxidative bleaching process. For the one hair sample without PTeCA in the distal segment, it could be that while first optically classified as cosmetically treated, no actual bleaching or hair dyeing was performed. As recently shown, gentle coloring procedures such as e.g. henna and tinting did not lead to a significant influence on PTCA contents[13] which would be accompanied by PTeCA formation. Additionally, this hair segment was located far from the hair root (> 40 cm from hair root) compared to the other treated segments (< 30 cm from hair root). A negative result due to a possible elimination of PTeCA over time can therefore not be excluded. Yet, segments representing distances of more than 40 cm from hair root are usually not analyzed during routine investigations so that the relevance of PTeCA elimination over such a long time can be neglected.

No significant changes between untreated and treated hair segments were observed for PTCA and isoPTCA in paired samples (see Figure S4 of the supplementary information). This is

unexpected at first as the PTeCA increase was significant and oxidative treatment therefore seems likely. It could be hypothesized that PTCA and isoPTCA indeed increased significantly, but are likely to decrease as exposure to weather (sunshine, rain), UV light and water facilitates a degradation or wash out over time[1,29]. In addition, hair structures are damaged through oxidative hair treatments provoking even more of a loss of analytes[28,30,31].

As PTeCA appears as a marker for eumelanin aging[16], formation without additional cosmetic treatments cannot be excluded. Though, longitudinal investigation of untreated hair samples showed that PTeCA stayed undetectable in all samples until the 4th segment (0-12 cm) (see Figure 5). As routine hair analysis usually investigates the first proximal 6 cm segments because the time resolution of a hair segment decreases with increasing distance from the hair root[1], the detection of PTeCA in routinely analyzed segments would not be expected (unless bleaching had taken place).

In contrast, investigation of PTCA and isoPTCA contents along the hair shaft showed a steady increase from proximal to distal segments in all three hair samples (see Figure 5). This new formation without any further aggressive cosmetic treatments is to be expected as the photodegradation of melanin by ultraviolet (UV) and visible light is widely described[17,26,32-35]. Wakamatsu *et al.* found that free PTCA increases with increasing length from the hair base through (photo)degradation or UVA-induced degradation of eumelanin in human hair[17]. Therefore, the longer the hair is exposed to sunlight, the higher the PTCA and isoPTCA concentrations should be. This influence would also result in fairly different levels of PTCA and isoPTCA in the proximal segments used for routine analysis.

Even though our study was not of quantitative nature, a high variability of PTCA and isoPTCA contents among hair colors and segments was observed. Interindividual differences

are therefore high. In a recently published study, PTCA concentrations above 20 ng/mg in authentic hair samples were stated to be highly indicative of oxidative treatment[13]. Nevertheless, it remains unclear which hair segments were used for the determination of PTCA contents in that study.

Taken together, the use of PTCA and isoPTCA as markers for oxidative hair treatment is possible. However, to establish reliable cut-off values and to systematically assess baseline PTCA and isoPTCA levels, a larger sample size is needed. In contrast, PTeCA proved as a promising marker as it remained undetectable in untreated hair samples up to 12 cm from hair root. It was only formed through oxidative treatment within the proximal segments which therefore circumvents a complex implementation of cut-off values. Yet, only bleaching was investigated within our study and the evaluation of PTeCA under different cosmetic hair treatments (semi/permanent dyeing, tinting, permanent waves and thermal hair straightening) should be part of future studies. However, formation of PTeCA using H_2O_2 under alkaline conditions regardless of dyeing or bleaching is to be expected.

Conclusion

We described the finding of PTeCA which was formed through oxidative hair treatment additionally to PTCA and isoPTCA. In this first study, it could be shown that PTeCA is not detectable in untreated proximal hair segments up to 12 cm length and is only formed through oxidation of eumelanin. Thus, the detection of PTeCA in the proximal 3 to 6 cm segment is highly indicative for hair manipulation by oxidative hair treatments. More studies in regard to absolute concentrations, limit of detection and matrix effects are a prerequisite to assess the suitability under routine conditions. The integration of PTeCA into routine analytical

procedures might represent an important tool to monitor the occurrence and prevalence of adulteration attempts through hair bleaching.

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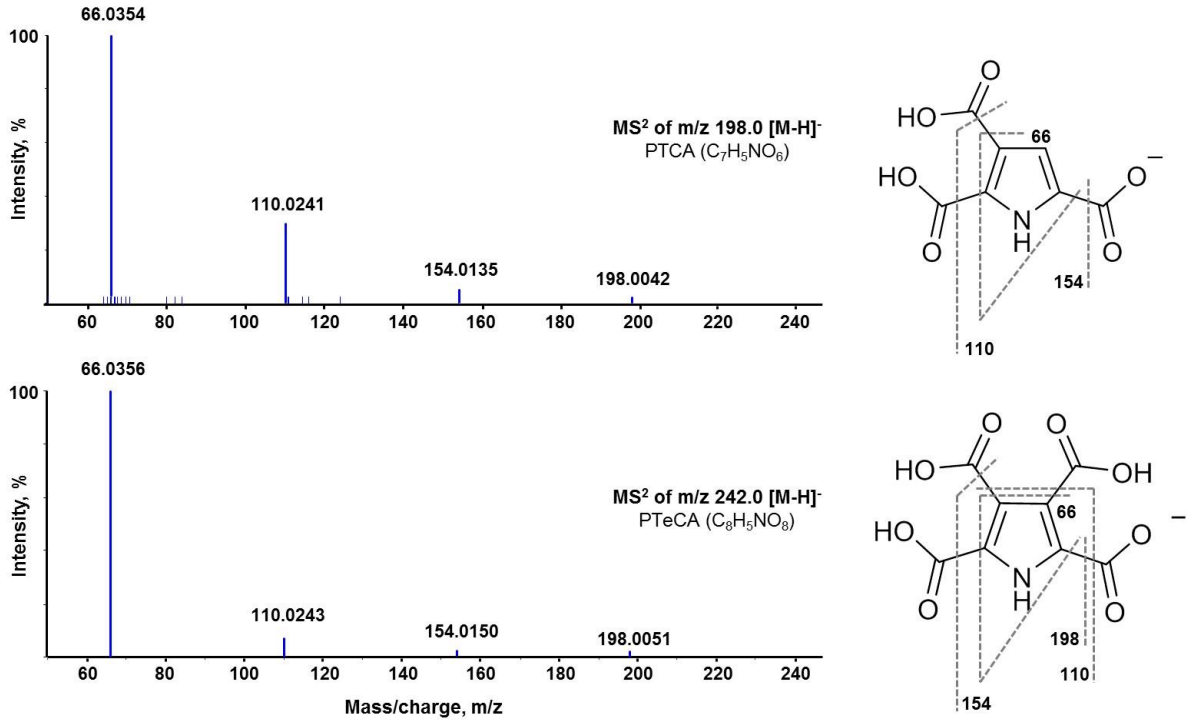

Fig. 1 QTOF MS/MS spectra with accurate fragment masses of the respective deprotonated molecular ions of PTCA and PTeCA, used for identification (collision energy 35 eV, collision energy spread ± 15 eV).

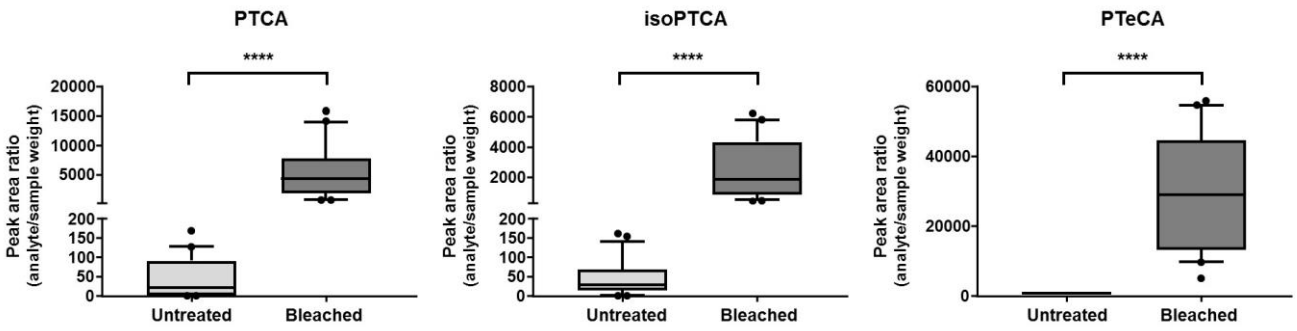

Fig. 2 Box plots for PTCA, isoPTCA and PTeCA. Analyte peak area ratios (compound/sample weight) are depicted for untreated hair samples (light grey) and hair samples after treatment with 9% H_2O_2 for 30 min (dark grey), n=21 for each group (experiment 1). Statistical analysis performed with a paired t-test: $p < 0.0001$ (****).

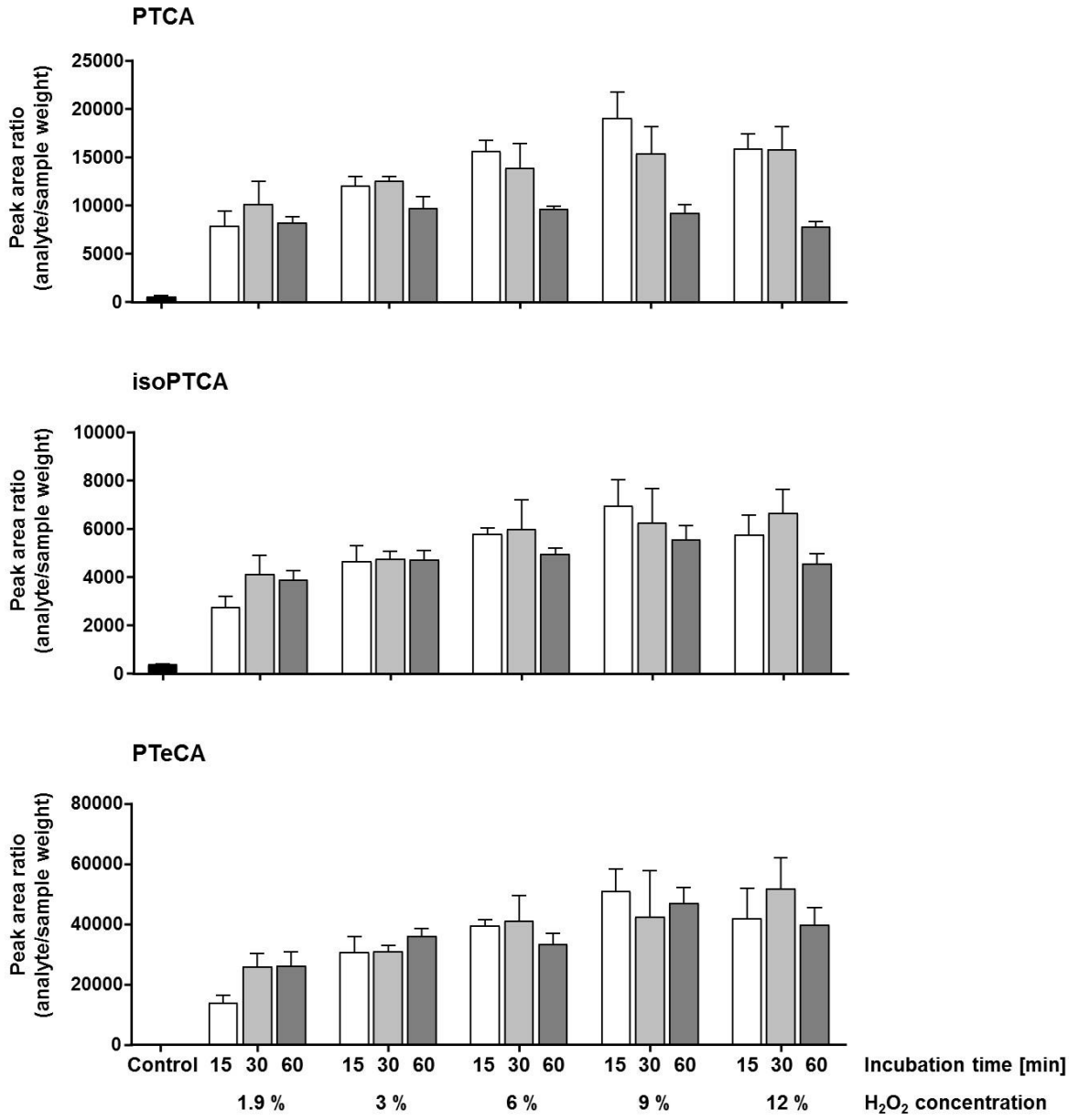

Fig. 3 Mean and SD (n = 3) of analyte peak area ratios (y- axis: compound/sample weight) of PTCA, isoPTCA and PTeCA in dependence of increasing H_2O_2 concentrations (1.9 %, 3 %, 6 %, 9 % and 12 %) and incubation times (15 min, 30 min 60 min) (x-axis) (experiment 2).

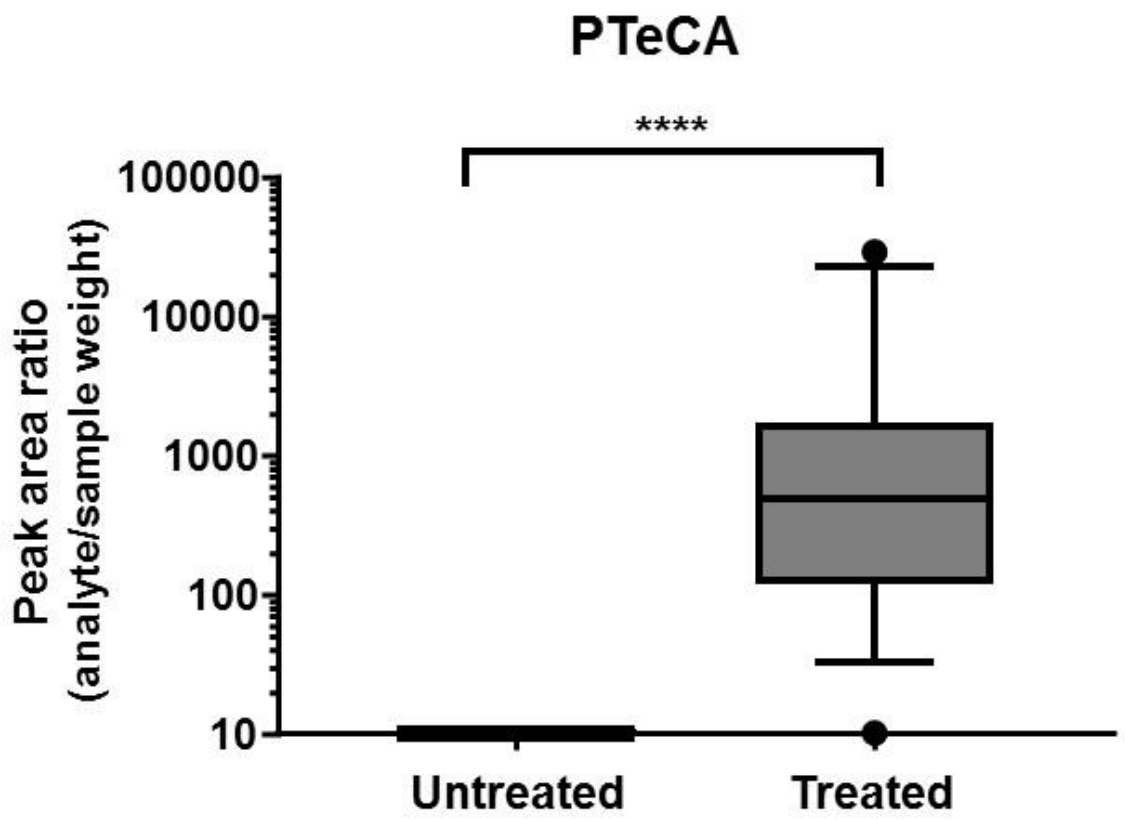

Fig. 4 Box plots from authentic hair samples, illustrated for PTeCA. Depicted are analyte peak area ratios (compound/sample weight) of 12 untreated hair samples (analysis of the proximal 3 cm segment) and 13 treated hair samples (proximal 3 cm segment: n = 2; distal segments: n = 11) (experiment 3). Statistical comparison using the non-parametrical Mann-Whitney-U test: $p < 0.0001$ (****).

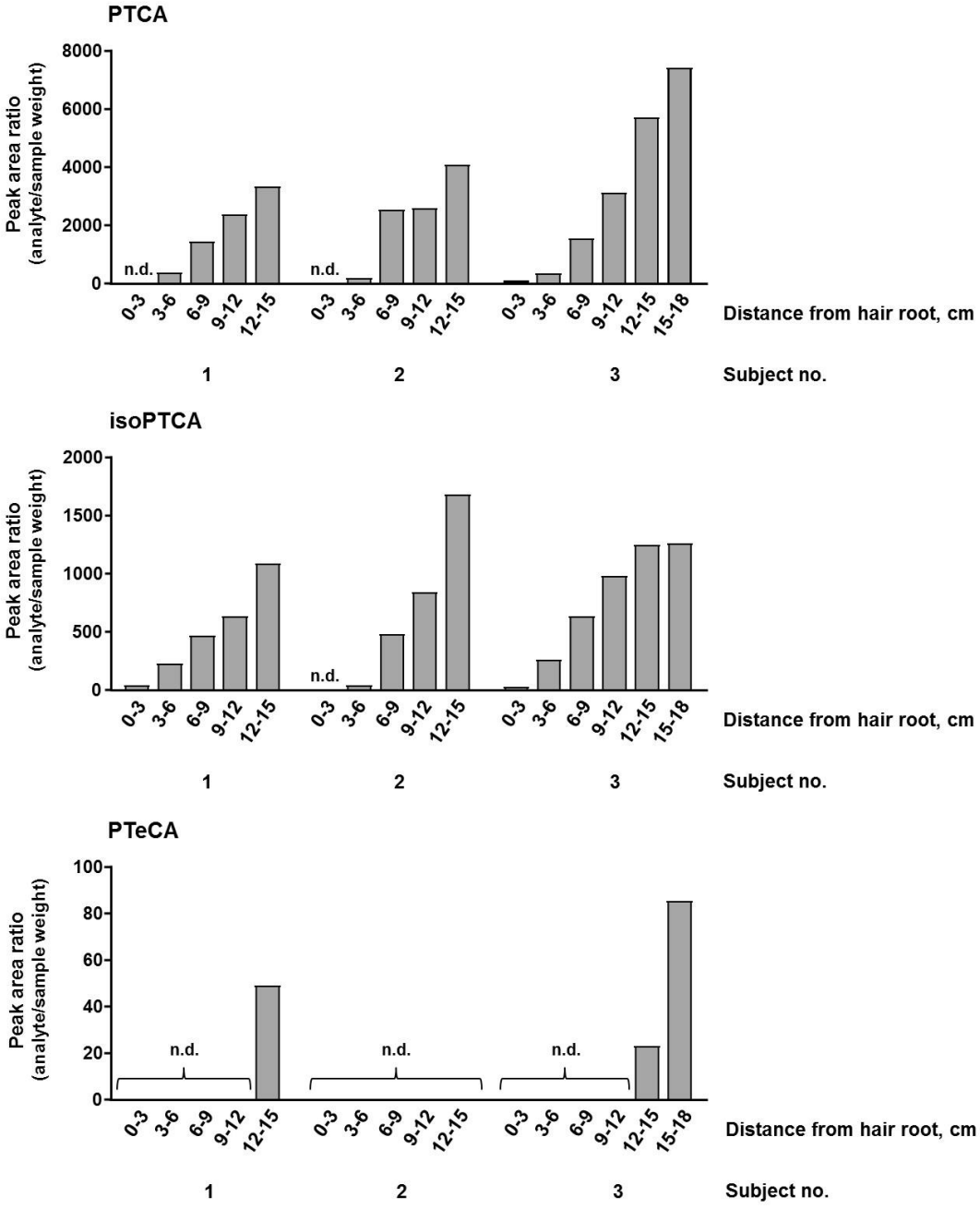

Fig. 5 Analyte peak area ratios (compound/sample weight) detected in cosmetically untreated hair strands (n = 3). Investigation of PTCA, isoPTCA and PTeCA in 3 cm long hair segments (experiment 4). n.d. = not detected. (Please, take note of the different scaling of the y-axes.)

A new oxidation marker for hair adulteration: detection of PTeCA (1H-pyrrole-2,3,4,5-tetracarboxylic acid) in bleached hair

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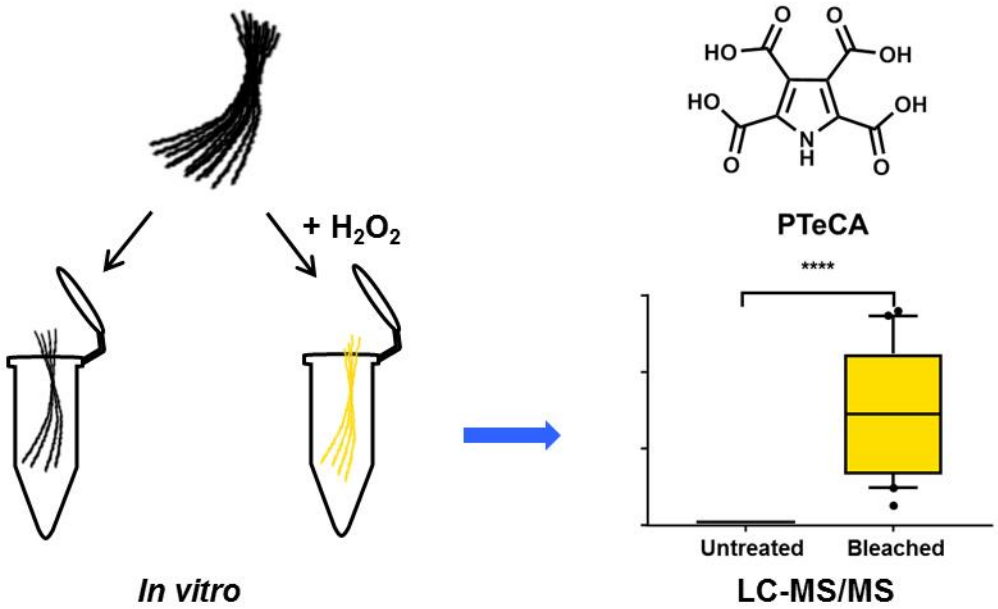

The eumelanin degradation product 1H-pyrrole-2,3,4,5-tetracarboxylic acid (PTeCA) was formed after *in vitro* bleaching of hair samples. Detection of PTeCA in hair samples provides a strong indication of oxidative hair treatment without necessitating cut-off values.